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Development of an antigen microarray for high throughput monoclonal antibody selection

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ABSTRACT

Monoclonal antibodies are valuable laboratory reagents and are increasingly being exploited as therapeutics to treat a range of diseases. Selecting new monoclonal antibodies that are validated to work in particular applications, despite the availability of several different techniques, can be resource intensive with uncertain outcomes. To address this, we have developed an approach that enables early screening of hybridoma supernatants generated from an animal immunised with up to five different antigens followed by cloning of the antibody into a single expression plasmid. While this approach relieved the cellular cloning bottleneck and had the desirable ability to screen antibody function prior to cloning, the small volume of hybridoma supernatant available for screening limited the number of antigens for pooled immunisation. Here, we report the development of an antigen microarray that significantly reduces the volume of supernatant required for functional screening. This approach permits a significant increase in the number of antigens for parallel monoclonal antibody selection from a single animal. Finally, we show the successful use of a convenient small-scale transfection method to rapidly identify plasmids that encode functional cloned antibodies, addressing another bottleneck in this approach. In summary, we show that a hybrid approach of combining established hybridoma antibody technology with refined screening and antibody cloning methods can be used to select monoclonal antibodies of desired functional properties against many different antigens from a single immunised host.

1. Introduction

The high binding affinity and specificity of monoclonal antibodies for their targets have made them invaluable tools for biomedical research and an increasingly important class of drugs that have been exploited to treat a range of diseases [1,2]. To select new monoclonal antibodies to a defined antigen, host animals are immunized and the resulting antibody-secreting B-lymphocytes are fused to a myeloma cell line to create a hybridoma. Hybridomas that secrete monoclonal antibodies of the required properties are selected so that they can be cultured indefinitely to provide large amounts of antibody as necessary [2,3]. While well-established, selecting monoclonal antibodies using this approach has several limitations that have made selecting monoclonal antibodies to multiple different antigens in parallel difficult. The limitations for scaling this approach include the use of laboratory animals, with standard protocols typically recommending immunising several animals per target antigen. Furthermore, because of the additional chromosomes, hybridomas are genetically only metastable, often necessitating the repeated cellular cloning of the hybridoma cell line which can be lengthy and labour intensive. Finally, it takes up to 2 weeks after the cellular fusion procedure before single hybridomas have divided to form a colony that is large enough to secrete sufficient amounts of antibody to permit robust screening.

Because of the usefulness of monoclonal antibodies, a wide range of different techniques for selecting them have been developed that bypass some or all of these limitations. Approaches using libraries of antibody-based binding reagents and *in vitro* selection methods such as phage display [4] and similar methods [5] have been particularly successful and obviate the need for animals. The requirement to create and culture hybridomas can also be circumvented by sorting individual antigen-specific B-lymphocytes and amplifying the regions encoding the rearranged antibody light and heavy chain regions by single cell RT-PCR; once cloned, antibodies can be expressed recombinantly by transfecting mammalian cell lines [6]. Variations include B-cell panning [7], lithographic methods of single cell incubation [8] or spotting of single cells onto an antigen coated chip [9], each of which have their own advantages for certain applications.

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While these alternative methods have specific advantages, animal immunisation and the generation of hybridomas have two important features. Firstly, the affinities of antibodies raised *in vivo* are often higher than those from *in vitro* selection methods due to the process of somatic hypermutation; and secondly, hybridoma colonies typically secrete sufficient amounts of antibody to permit some functional screening so that subsequent cloning efforts are focussed only on antibodies that have the required immunological or biochemical properties. With these points in mind, we developed a convenient method of selecting monoclonal antibodies against multiple antigens immunised as a pool into a single animal [10]. This hybrid approach ensured high-affinity antibodies were elicited, and that some hybridoma supernatant was available for screening to identify antibodies with desired functional properties prior to cloning. Selected antibodies were cloned by amplification of the rearranged antibody light and heavy chains by RT-PCR from the hybridomas, and ligated into a single expression plasmid that could be used to express the antibodies recombinantly [10]. Using this approach, we were able to immunise and screen up to five different antigens per mouse, a number that was restricted by the small volume (~200 μl) of available antibody-containing supernatant per hybridoma and our use of a standard ELISA in our antibody selection screen. Because, in principle, antibodies to more antigens could be obtained from a single mouse, we sought to reduce the amount of hybridoma supernatant required for initial antibody screening and address an additional bottleneck in this method: the identification of functional antibody-encoding plasmids.

We now describe the development and use of a protein microarray that permits the screening of up to 100 different antigens with small volumes of undiluted hybridoma tissue culture supernatant which significantly increases the number of antibodies that can be cloned from a single mouse in parallel. In addition, we describe a refinement using the small scale transfection of HEK293 cells which facilitates the identification of functional antibody expression plasmids. Together, these refinements reduce the number of animals required for generating monoclonal antibodies and vastly increase the potential throughput of this method of monoclonal antibody generation.

2. Materials and methods

2.1. Recombinant protein production and purification

The extracellular domains of zebrafish proteins used in this study were expressed as monobiotinylated proteins using mammalian cells. Expression plasmids were made from published resources [11–13] by subcloning the NotI/AscI enzyme flanked ectodomains [14] into a plasmid containing a C-terminal rat Cd4 domains 3 and 4, an enzymatically biotinylatable peptide sequence, and a 6 His-tag [11,15]. The ectodomains of zebrafish proteins and recombinant antibodies were expressed by transient transfection of either HEK293E [16] or F (Invitrogen) cells. To monobiotinylate proteins during expression, cells were co-transfected with a plasmid encoding a secreted *Escherichia coli* BirA enzyme [11,15]. Supernatants were harvested after 6 days, filtered, and purified using Ni^{2+}-NTA Sepharose (Invitrogen) [17]. Proteins were assessed by SDS–PAGE and protein biotinylation confirmed by ELISA [15]. Recombinant antibodies were purified using protein G columns (GE Healthcare) [10].

2.2. Immunizations and hybridoma generation

Six-week-old Balb/c mice were immunised intraperitoneally with pools of up to twenty antigens (5 μg each) mixed with Gold's Adjuvant (Sigma) three times at 4 week intervals. Mice were given a final immunisation without adjuvant 3 days before the spleen was removed. Splenocytes (10^8) were fused to SP2/0 myeloma cells (10^7) in 50% PEG 1500 (Roche, Hertfordshire, UK) using standard procedures [10]. The hybridoma mixture was plated over twelve 96-well plates and supernatants were harvested after 10–14 days for screening.

2.3. Printing of protein microarrays

Purified biotinylated proteins were spotted at the base of streptavidin-coated 96-well microtitre plates (NUNC Immobilizer, Thermo Scientific, Denmark) using a Microgrid II arrayer (BioRobotics) by direct contact printing using 0.2 or 0.4 mm solid printing pins. Printed plates were left unwashed and plates were stored at 4 °C unless described otherwise. For screening, hybridoma supernatants were added directly to the wells and a specific blocking step was found not to be required. A biotinylated anti-rat Cd4 antibody was used as a positive control and orientation marker.

2.4. Screening of protein microarrays

Hybridoma supernatants were added to the antigen arrays and incubated overnight at 4 °C. After washing with PBT (PBS + 0.1% Tween), arrays were incubated for 2 hours with a goat anti-mouse Alexa 488 secondary antibody (Invitrogen), washed in PBT, followed by a rabbit anti-goat Alexa 488 antibody (Invitrogen). This second amplification step, although not absolutely required, increased signals to permit direct visual screening using an epifluorescence microscope. In some cases, antibodies were detected with an anti-mouse Alexa 568 secondary antibody (e.g. Fig. 1C) or an anti-mouse alkaline phosphatase secondary followed by NBT/BCIP (Roche) as a precipitating colourimetric substrate (e.g. Fig. 1B). Antigen arrays were analysed on a Leica MZ 16 FA microscope, images captured using an Axiocam HRC (Zeiss) and adjusted for brightness and contrast with Adobe Photoshop CS4.

2.5. Identification of functional recombinant antibody plasmids by transient transfection

The rearranged variable heavy and light chain antibody regions were amplified from hybridoma cDNA by RT-PCR and recombined into one PCR product using a fusion PCR-based strategy that, after cloning into a suitable expression vector, enables the expression of a recombinant antibody from a single expression vector, as described [10]. In brief, the fused PCR products from each hybridoma were ligated into an expression vector and the mixture used to transform chemically competent bacteria. Plasmids were purified from 96 bacterial colonies for each hybridoma. Suspension cultures (1 ml) of HEK293 cells were transfected in 24-well plates with 2.5 μg plasmid DNA using Metafectin (Biontex Laboratories), supernatants harvested after 3 days and tested by ELISA. The plasmids encoding the antibodies described in the study are openly available from Addgene (http://www.addgene.org).

3. Results

3.1. The printing of protein arrays in 96-well plates permits the parallel screening of a large number of antigens

To increase the number of monoclonal antibodies that could be selected in parallel from a single mouse immunized with multiple antigens, we aimed to print small protein microarrays at the base of 96-well microtitre plates (Fig. 1A). We have developed an approach that enables the expression of enzymatically